AD_____

AWARD NUMBER DAMD17-96-1-6008

TITLE: DNA Topoisomerase I-Targeted Therapy for Breast Cancer

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REPORT DATE: June 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 1998	Annual (6 May 97 - 5 May 98)

4. TITLE AND SUBTITLE
DNA Topoisomerase I-Targeted Therapy for Breast Cancer

5. FUNDING NUMBERS
DAMD17-96-1-6008

6. AUTHOR(S)
Von Hoff, Daniel D., M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
CTRC/Institute for Drug Development
San Antonio, Texas 78229-3264

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The specific aim of this work is to develop camptothecin (CPT) analogs for treatment of women with breast cancer. We evaluated these analogs using three biochemical tests: (i) inhibition of growth of breast cancer cells, (ii) induction of topoisomerase I-mediated cleavage of DNA, and (iii) reversal of DNA cleavage. Our results indicate that the 10,11-methylenedioxy CPT analog was effective in all three tests. Further, this analog was effective against MX-1 and MDA-231 human breast cancer xenografts growing in nude mice. The 10,11-methylenedioxy analog appears to be the best choice among the analogs examined, and we are currently pursuing moving this compound into clinical studies.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
28

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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5/30/95

PI - Signature Date

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A. INTRODUCTION

A.1 Cancers, Camptothecin and Topoisomerase I

Cancer is the second leading cause of death (just behind cardiovascular diseases) in the United States. More than one million people are diagnosed with cancer and about half of them die each year in the United States. Surgery, radiation and chemotherapy are the three major modalities for the treatment of cancer. Surgery and radiation are very effective in the removal of tumor locally. Since cancer is a disseminated disease, chemotherapy is generally needed for the treatment of metastatic diseases. Although there have been many advances in cancer treatments and many success stories, the most common cancers — breast, colon, lung and prostate — remain rather refractory to treatment.

In 1966, Dr. Monroe Wall and colleagues discovered that camptothecin (CPT) was the active principal of the extract from the stem of the Chinese tree *Camptotheca acuminata* [1]. CPT had significant antitumor activity against L1210 murine leukemia [1]. Early clinical trials with CPT in the late 1960s showed that this plant alkaloid had activity against a variety of solid tumors [2-4]. However, further development was discontinued because of unpredictable and severe myelosuppression, gastrointestinal toxicity and hemorrhagic cystitis.

Further development of topoisomerase I (topo I) inhibitors for cancer therapy was stimulated by the characterization of CPT as a specific topo I inhibitor [5, 6] Eukaryotic topo I engages DNA in a cycle of breakage and religation steps. These topological adjustments are important in local DNA unwinding during DNA synthesis, repair, and transcription. Topo I relaxes DNA supercoiling by making transient single-strand breaks [7, 8]. These breaks are coupled with the transient formation of a covalent DNA-enzyme intermediate termed the cleavable complex [5]. CPT specifically and reversibly stabilizes cleavable complexes by inhibiting their religation. The mechanism of CPT cytotoxicity is thought to be the consequence of a collision between moving replication forks and CPT-stabilized cleavable DNA-topo I complexes [9-11].

A.2 Irinotecan and Topotecan

There are several topo I inhibitors at various stages of clinical development. Of these, irinotecan (CPT-11) and topotecan have been approved by the Food and Drug Administration. CPT-11 is a pro-drug and must be converted to SN38 by carboxylesterase to exert its antitumor activity [12, 13]. Neutropenia and diarrhea are the dose-limiting toxicities of CPT-11 (depending on the schedule) [14, 15]. Thrombocytopenia is the dose-limiting toxicity for topotecan [16]. CPT-11 has shown antitumor activity in phase II trials for patients with carcinoma of the colon, lung, cervix, and ovary, as well as for patients with non-Hodgkin's lymphoma. Topotecan has demonstrated clinical antitumor activity against advanced small cell lung and ovarian cancers [14, 15]. Irinotecan has recently been approved for the second-line treatment of metastatic colorectal cancer in the U.S. and Europe and for other additional indications in Japan. Topotecan was approved for the treatment of patients with ovarian cancer that is refractory to the platinum analogs.

A.3 Hypothesis/Purpose

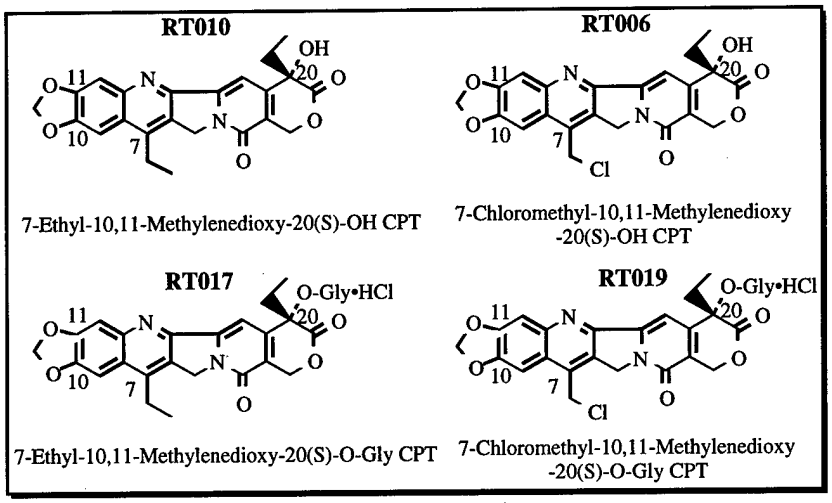
Our hypothesis is that CPT analogs will have better clinical antitumor activity against breast cancer than irinotecan or topotecan if those CPT analogs (i) have a more stable lactone ring, (ii) are capable of forming an irreversible cleavable DNA-topo I complex, and/or (iii) are not cross-resistant to irinotecan or topotecan. Toward these goals, a number of novel CPT analogs were synthesized by Research Triangle Institute and given to the Institute for Drug Development for biochemical, *in vitro*, and *in vivo* testing. Here, we highlight the work peformed during year two of the award.

A.4 Previous Results

In our last report, we designed a water soluble camptothecin analog (RT019, or CMMD•Gly), 7-chloromethyl-10,11-methylenedioxy-20(S)-O-glycinate CPT•HCl as a potential third generation of camptothecin analogs for the treatment of patients with refractory tumors. This compound processes two essential groups: (i) a 20(S)-O-glycinate group which could protect the E-ring lactone from

undergoing pH-dependent hydrolysis and (ii) a 7-chloromethyl group which can trap DNA-topo I cleavable complexes irreversibly.

Fig.1 Chemical Structure of RT006, RT010, RT017, and RT019



We compared the biological activity of RT019 (CMMD•Gly) to three related compounds (i) RT006 (CMMD), 7-chloromethyl-10,11-methylenedioxy-20(S)-OH CPT, a parent compound of RT019, (ii) RT017 (C2CPT•Gly), 7-ethyl-10,11-methylenedioxy-20(S)-O-glycinate CPT•HCl, a counterpart of RT019, which could not trap DNA-topo I cleavable complexes irreversibly, and (iii) RT010 (C2CPT), 7-ethyl-10,11-methylenedioxy-20(S)-OH CPT, a parent compound of RT017. In addition, we compared the activity of these four CPT analogs with that of irinotecan and topotecan.

We found that the growth inhibitory activities of the 20(S)-O-glycinate and 20(S)-OH CPTs are remarkably similar (RT010 *vs.* RT017 and RT006 *vs.* RT019). In the growth inhibitory activity studies, the cells were incubated with testing agents continuously for 3-6 days, and the glycinate group may have been hydrolyzed in the cultured medium. Therefore, the glycinate and its parent compound exerted a similar growth inhibitory activity. On the other hand, we were surprised to find that 7-ethyl substituted analogs (RT010 and RT017) demonstrated more potent growth inhibitory activity than their corresponding 7-chloromethyl-substituted analogs (RT006 and RT019). This was somewhat perplexing, since we have speculated that compounds with a 7-chloromethyl group that can form an irreversible DNA-topo I complexes may be more potent antitumor agents. Therefore, it would be very important to understand why compounds which theoretically do not form an irreversible cleavable complex are more potent antitumor agents.

Since tumor cells were incubated continuously with testing agents in the growth inhibitory studies, it was difficult to distinguish the antitumor potential of CPT analogs which trap DNA-topo I cleavable complexes reversibly or irreversibly. We used a clonogenic survival assay to address whether compounds with the ability to trap DNA-topo I cleavable complexes irreversibly were better antitumor agents. In the clonogenic survival assay, the cells were treated with test agents for 1 hr and were replated in drug-free medium. We found that CPT analogs (RT006 and RT019), which trap DNA-topo I cleavable complexes irreversibly, produced more extensive tumor cell kill than the CPT analog (RT017) which traps DNA-topo I cleavable complexes reversibly.

The experiments documented last year suggested that our compounds RT017 and RT019 and their hydrolyzed metabolites are more potent *in vitro* against breast cancers than those topo I inhibitors currently being used clinically. This suggested that these agents may be powerful new antitumor agents for treatment of patients with breast cancer, and consequently, other analogs based on these compounds might also be effective new antitumor agents. Our experiments over the last 12 months focused on two aspects necessary to begin to examine these agents for possible clinical use: (1) mechanistic studies of the cellular toxicities of the compounds, and (2) the activities of the compounds in *in vivo* models of breast cancer.

B. BODY

B.1. Synthetic Studies

The following compounds have been made as described in the original Appendix 7D, Statement of Work from our grant proposal. A copy of this Statement and associated charts A-C are attached at the end of this report as Appendix 2.

B.1.a. Compounds A1-A6.

All compounds shown as A1-A6 (Appendix 2, Chart A) have been prepared.

B.1.b. Compounds A7-A10.

The corresponding 20-gylcinate-HCl esters A-7 to A10 have all been prepared. In addition, the glycinate esters of compounds of A3 and A4 have been prepared. Moreover, a study of the hydrolysis of glycinate esters in various media has been instituted; cf. section B.1.g. For these studies, quantitative HPLC assays for the glycinate ester and for open ring lactone forms have been developed; cf. section B.1.g for a full description. For this purpose, the 20-glycinate ester-HCl of camptothecin has been prepared, and in addition the N-methyl and N-dimethyl glycinate esters have been prepared. The general syntheses of the above compounds and the corresponding glycinate esters have been described in detail (M. E. Wall et al., J. Med. Chem., 36, 2689, 1993). None of compounds A-11 to A-17 have been prepared.

B.1.c. 7-Halomethylcamptothecin Analogs

The general preparation for these compounds is described in Chart B (Appendix 2), section 3-B.3. Compounds B1 and B2 have been prepared. The bromomethyl analog, compound B2, is new, and its preparation by a novel route is shown in the attached Chart 1 (Appendix 1), compound 3. In addition, 7-bromomethylcamptothecin, Chart 1, compound 1, and 10-hydroxy-7-bromomethylcamptothecin, Chart 1, compound 2, have both been prepared for the first time. The 10,11-methylenedioxychloro and bromo compounds have been shown to alkylate DNA and hence may be of major interest.

B.1.d. Synthesis of CPT Analogs with E-Ring Ketone (Grant Chart C)

This synthesis has been attempted but to date has been unsuccessful. The method that has been attempted with some partial success is shown in the attached synthesis of the E-ring ketone analog of CPT (Appendix 1, Chart C2). In this procedure, camptothecin was converted with ethyl amine to the corresponding amide (**1**). The primary hydroxyl moiety, which is formed by opening of the lactone ring, was acetylated yielding **2** and then converted to the corresponding malonate diester (**3**). However, attempts to hydrolyze the amide to obtain the corresponding ethyl ester (**4**) have failed; i.e., the hydrolysis of the amide group has been unexpectedly very difficult. We still believe this method has promise and will continue to work on it in year 03 because it will require only two more relatively simple steps to obtain the E-ring ketone (**6**). We plan to continue work on all of these lines in year 03, particularly based on finding methods of hydrolyzing the amide initially formed from camptothecin.

B.1.e. Preparation of Larger Samples for In Vivo Testing

We have prepared in several hundred milligram quantities compound A1, Chart A (Appendix 2), section 3.B.2 and the corresponding glycinate ester A2. In addition, we have prepared sizeable compounds from Chart B1 (Appendix 2), 10,11-methylenedioxy-7-chloromethyl-20(S)-camptothecin and the corresponding 20(S)-glycinate ester hydrochloride.

B.1.f. Studies Related but not Specifically under Statement of Work, Appendix 7, Section D

Preparation of Difluoromethylenedioxy-(S)-Camptothecin Analogs.

At the end of the second year and extending well into a third year of this DOD sponsored program, the RTI group has investigated the synthesis of difluoromethylenedioxy-camptothecin and analogs. The

synthesis of this compound is shown in Chart 2 and various analogs that might be prepared from it in Chart 3. At this stage, these compounds will require considerable investigation; i.e. activity against various tumor cells, topoisomerase inhibition, time for reversal of topo I inhibition, etc. At this point, we have obtained preliminary evidence showing that the base compound is a potent topoisomerase I inhibitor and also has cytotoxicity greater than camptothecin and possibly of the same order as the 10,11-methylenedioxy analog. The potential advantage of the difluoro compound is that the methylenedioxy group cannot be metabolized and give rise to possibly toxic components as could occur with 10,11-methylenedioxy-20(S)-CPT, which can metabolize to formic acid or formaldehyde, both toxic.

B.1.g. Hydrolysis and Stability of Camptothecin, Camptothecin-20(S)-Glycinate Ester Hydrochloride, and Camptothecin-20(S)-Ester Dimethyl Glycine Ester Hydrochloride, a Study by HPLC Methodology

In the last several years, there has been great interest aroused in the stability of the camptothecin lactone system of various analogs. It has been suggested that studies of the inhibition of various cancer tumors in mice might not give the same effects in humans because of the differences of stability of CPT and various analogs in man, e.g. human plasma vs. mice plasma. Accordingly, a study has been made of camptothecin, camptothecin glycinate hydrochloride ester, and camptothecin dimethyl glycinate ester hydrochloride in human plasma. The methodology for each study was similar: in general, the compounds were incubated in human plasma for various time periods at 37.5 °C with stirring. Aliquots were removed at selected intervals. Analysis of the aliquots was performed with a SPE C18 column. The following results were obtained for CPT:

time	peak area (CPT)	peak area (unknown)
0	2939	222
15 min	2486	262
30 min	1213	407
60 min	91	538
24 hr	0	481

CPT was completely converted to another form in a period of 1 hour. The unknown compound was not identified, but is most likely the lactone open-ring form of the drug. Thus, CPT is completely hydrolyzed or degraded in 1 hour when incubated with human plasma.

The next data are shown for camptothecin-20(S)-glycinate hydrochloride. When the hydrochloride is incubated in plasma at pH 7.5, it is rapidly converted to the free base--the hydrochloride being completely neutralized. Although the data are limited, within 1 hour the area of the glycinate had decreased greatly. The ester was hydrolyzed and converted to camptothecin.

time	peak area (CPT•Gly)	peak area (CPT)
0	2457	2123
60 min	933	7353

In the final study, camptothecin-20-(S)-dimethyl-glycinate ester hydrochloride was incubated with human plasma. The aliquots taken at different times from the plasma were divided into two groups prior to cleanup. One group (E1) was applied directly to the column. The other group (E2) was acidified prior to application to the column to give the amount of total drug. As shown here, the E1 sample, which is primarily unchanged ester, decreased slowly with time.

time	peak area (glycinate) E1	peak area (CPT) E1	peak area (Unknown) E1	peak area (CPT) E2	peak area (Unknown) E2
0	4204	185	112	45	2768
24 hr	3810	120	718	615	1565
48 hr	3294	104	1042	1281	344
96 hr	1683	387	†	†	†

†Appearance of several large, unresolved peaks.

B.2 Biochemical Studies of CPT analogs

Due to the potency of the RT010 and RT006 compounds, a series of 10,11-methylenedioxycamptothecin analogs (based on A-10 in Chart A of our grant) were synthesized and tested for in vitro activity in three ways (i) against MDA-231, BT-20, and ZR-75 breast cancer cell lines, (ii) for their ability to induce topo I-mediated cleavable complexes in plasmid DNA, and (iii) the reversibility of the complexes.

The analogs are designated as follows:

Abbreviation	Compound Name
CPT(S)	Camptothecin
MDCPT(S)	10,11-methylenedioxycamptothecin
CPT-11	CPT-11
OHCPT	10-hydroxycamptothecin
NH2CPT	10-amino-7-n-butylcamptothecin
OHC1CPT	10-hydroxy-7-methylcamptothecin
OHC2CPT	10-hydroxy-7-ethylcamptothecin
OHC3CPT	10-hydroxy-7-n-propylcamptothecin
OHC4CPT	10-hydroxy-7-n-butylcamptothecin
OHC5CPT	10-hydroxy-7-n-pentylcamptothecin
OMeC1CPT	10-methoxy-7-methylcamptothecin
OMeC2CPT	10-methoxy-7-ethylcamptothecin
OMeC3CPT	10-methoxy-7-n-propylcamptothecin
NNDE•Gly	CPT-20-N,N-diethylglycinate•HCl
MDNNDE•Gly	10,11-MD-20-N,N-diethylglycinate•HCl
Pyrrazalo	Pyrarrazole CPT
MDCPT(R)	10,11-methylenedioxy-20(R)-camptothecin
CPT(R)	20(R)-camptothecin
CMMD	7-chloromethyl-10,11-methylenedioxy-CPT
CMMD•Gly	7-chloromethyl-10,11-MD-CPT-20-Glycinate

Table 1. Abbreviations used to Designate Camptothecin Analogs.

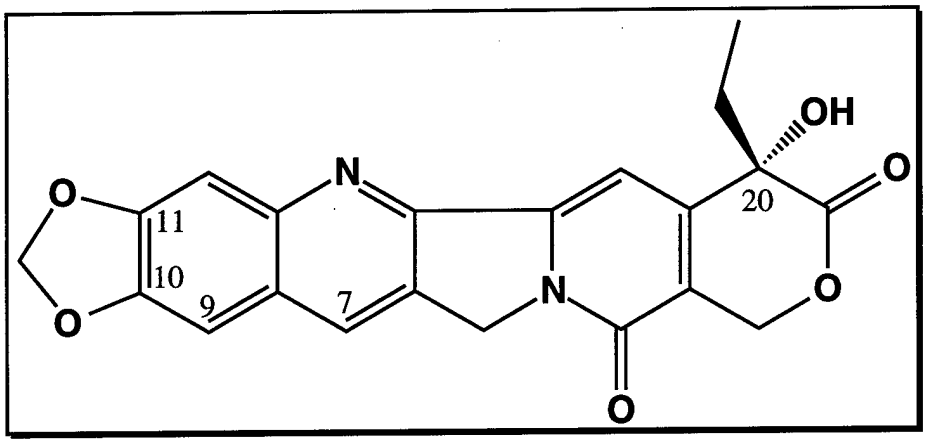


Fig. 1. 10,11-methylenedioxycamptothecin (MDCPT)

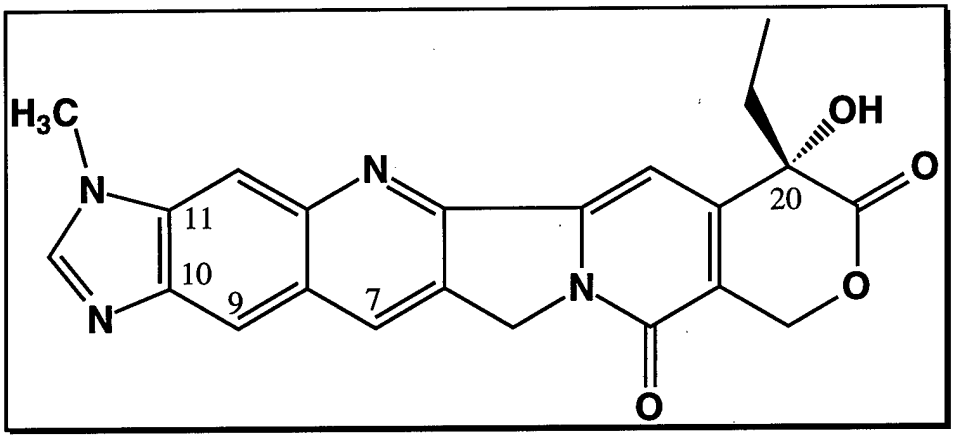


Fig. 2. Pyrrazalo CPT

B.3 Cell Culture

Human breast carcinoma lines MDA-23, ZR-75, and BT-20 were maintained in IMEM supplemented with 10% fetal bovine serum. ZR-75 media also contained 10 µg/ml bovine insulin. All lines were maintained at 37 °C in 5% CO_2 incubators until plated for use in MTT assays.

B.4 *In Vitro* Growth Inhibitory Activity

Exponentially growing cells (1-2 x 10^3 cells, unless otherwise specified) in 0.1 ml medium were seeded on day 0 in a 96-well microtiter plate. On day 1, 0.1 ml aliquots of medium containing graded concentrations of test analogs were added in duplicate to the cell plates. After incubation at 37 °C in a humidified incubator with 5% CO_2-95% air for 3 days, the plates were centrifuged briefly and 100 µl of the growth medium was removed. Cell cultures were incubated with 50 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide [MTT, 1 mg/ml in Dulbecco's phosphate buffered saline (PBS)] for 4 hr at 37 °C. The resulting purple formazan precipitate was solubilized with 200 µl of 0.04 N HCl in isopropyl alcohol. Absorbance was monitored in a BioRad Model 3550 Microplate Reader at a test wavelength of 570 nm and a reference wavelength of 630 nm. The absorbance was transferred to a PC 486 computer. The IC_{50} values were determined by a computer program (EZ-ED50) (Perrella Scientific, Inc.) that fits all of the data to the following four-parameter equation:

$$Y = \frac{A_{max} - A_{min}}{1 + (X/IC_{50})^n} + A_{min} \qquad \text{eq. 1}$$

where A_{max} is the absorbance of control cells, A_{min} is the absorbance of cells in the presence of highest agent concentration, Y is the observed absorbance, X is the agent concentration, IC_{50} is the concentration of agent that inhibits the cell growth by 50% of control cells (based on the absorbance) and n is the slope of the curve.

B.5 Cleavable Complex Formation by CPT Analogs

Our reaction mixtures consisted of: 2μL of 10X Enzyme reaction buffer (10 mM Tris-HCl pH 7.9, 1 mM EDTA, 0.15 M NaCl, 0.1% BSA, 0.1 mM Spermidine, 5% Glycerol); 250 ng pBR322 plasmid DNA (Gibco BRL); 4 units Human Topoisomerase I enzyme (Topogen Inc.); drug of interest; and H_2O (20 μL final volume), and were assembled in ice.

Each mixture was incubated at 37°C for 30 minutes, then terminated by the addition of 2 μL of 10% SDS and 2 μL of 0.5 mg/mL Proteinase K (Promega). The mixture was further incubated for another 30 minutes at 37°C, then treated with 2μL loading solution (25 % bromophenol blue, 50 % glycerol) and extracted with 20 μL CIA (Chloroform: Isoamyl Alcohol, 24:1). Following the CIA extraction, the resulting sample was analyzed by electrophoresis for 16 hours at 30 volts on a 1% agarose gel in 1% TAE buffer (pH 8.0, containing 0.5μg/ml Ethidium Bromide). After electrophoresis, the gel was stained with 1:10 000 dilution of SYBR Green (Molecular Probes) in TE buffer (pH 8.0) and photographed under transillumination with 300 nm UV light. The resulting photograph was scanned using a Polaroid Photopad scanner and the nicked DNA (Form II DNA) band quantified using NIH Image 1.6 software. A linear relationship between the amount of DNA present and the signal generated was established by quantifying varying concentrations of supercoiled DNA.

Dose response data were fitted to a simple E_{max} model according to:

$$\text{Cleaved Complex} = \frac{E_{max}[Drug]}{EC_{50}[Drug]} \qquad \text{eq. 2}$$

where [Drug] is the molar concentration of analog, Emax is the maximal cleaved DNA signal, and EC_{50} is the concentration of drug required to produce 50% of the maximal response. Data were fitted to this equation using the nonlinear least squares routine in Kaleidagraph (Synergy Software, Reading, PA).

B.6 Reversal of Cleavable Complex Formation.

Reversal of the Topo I cleavage activity was accomplished by the addition of 100 fold excess of linear DNA to a reaction mixture containing drug at a concentration previously shown to inhibit Topo I activity . The reaction protocol consisted in the preparation of one 75 μL reaction mixture containing: 10X Enzyme reaction buffer (see above), 0.94 μg pBR322 plasmid DNA, CPT analog (at a concentration previously established to inhibit Topo I activity), 10 units Topo I enzyme and H_20 to 75 μL final volume. The reaction mixture was incubated for 30 minutes at 23°C. At the end of the incubation period one 5.0 μL aliquot was removed to represent the 0 sec control sample. A 100-fold excess of sonicated salmon sperm linear DNA (Gibco BRL, 10mg/mL) was added to the reaction mixture. Subsequent aliquots were removed at 0.5, 1, 2, 5, 10,15, 30, 45 and 60 minute intervals. The reaction was stopped by the addition of 11 μL H_20, 2 μL 10% SDS and 2 μL Proteinase K, then incubated at 23°C for another 30 minutes. The resulting samples were further mixed with 2 μL loading solution (see above) and analyzed by electrophoresis (19 hours at 22 volts) on a 1% agarose gel containing Ethidium Bromide (as in the cleavage assays).

Following electrophoresis, the gels were destained twice for 30 minutes in TE buffer (pH 8.0) then stained with a 1:10 000 dilution of SYBR Green solution in TE (pH 8.0) for 30 minutes. Polaroid photographs of the resulting gels were taken under 300 nm UV light and quantified in the same manner as for the cleavage assays. The percentage of DNA cleavage was determined based on the amount of Form II DNA present in the 0 sec control lane.

The time constants for repair of topo I-mediated strand breaks was fitted to the following equation:

$$\% \text{ Cleaved DNA} = A\exp(-kt) + Bt + C \qquad \text{eq. 3}$$

For some analogs, the exponential term was zero, and the data could be fitted to a simple linear model.

B.7 Results

B.7.a Growth Inhibitory Activity of CPT Analogs

The IC50 values for inhibition of growth for 3 breast cancer cell lines are given in Table 2. The majority of the new analogs were active at concentrations substantially lower than that for CPT. However, the Pyrrazalo analog was inactive, and it appears that the MDNNE•Gly was not effectively converted to MDCPT in tissue culture conditions, whereas the glycinate esters were.

All (S)-analogs bearing the 10,11-methylenedioxy substituent were highly active in all 3 cell lines. The 10-hydroxy or -methoxy groups produced approximately equal activity, which was about an order of magnitude higher than CPT. The length of the 7-alkyl substituent was not a significant factor in the toxicities of the 10-hydroxy or 10-methoxy analogs.

compound	MDA-231	BT-20	ZR-75
CPT(S)	28.58	25.74	10.88
MDCPT(S)	0.75	0.47	0.35
CPT-11	>1000	>1000	57.85
OHCPT	13.08	8.50	0.79
NH2CPT	0.83	0.67	0.08
OHC4CPT	0.84	1.20	0.08
OHC2CPT	2.52	2.23	0.32
OHC1CPT	2.83	1.26	0.11
OHC3CPT	4.18	3.10	0.29
OHC5CPT	1.50	0.83	0.13
OMeC1CPT	1.51	0.21	0.09
OMeC2CPT	4.92	0.90	0.29
OMeC3CPT	4.38	0.58	0.21
NNDE•Gly	40.24	12.57	6.07
MDNNDE•Gly	>1000	>1000	
Pyrrazalo	>1000	>1000	36.87
MDCPT(R)	3.43	0.88	0.42
CPT(R)	>1000	>1000	28.52
CMMD•Gly	3.87	1.07	0.23

Table 2. IC50 values for CPT Analogs Against Breast Cancer Cell Lines

B.7.b Inhibition of the Topo I Catalytic Activity by CPT Analogs

Using the cell-free biochemical assay, we determined whether the CPT analogs could induce topo I-mediated DNA cleavage in pBR322 supercoiled plasmid DNA. All agents that were active against

the 3 cell lines were also highly potent in inducing DNA cleavage. On average, the 10,11-methylendioxy analogs were 10- to 100-fold more potent in inducing DNA cleavage as compared to camptothecin alone. Neither the Pyrrazalo nor the MDNNDE•Gly analog showed any ability to induce DNA cleavage. However, the NNDE•Gly analog did show some activity, suggesting this analog was converted to CPT during the topo I cleavage assay. At present, we are repeating the OMeC2CPT experiment, as the data for this analog was not similar to the other analogs tested, and could not be fitted to the simple E_{max} model.

		EC 50 Equation				
Cpd. Abrev.	**Cpd. #**	**E max**	**EC 50**	**Error**	**R value**	**Comments**
CPT(S)	1	1281.8	44.43	17.66	0.981	
MDCPT(S)	2	597.04	0.3283	0.15	0.92	
CPT-11	3	31.51	0.25982	0.8247	3.63E-01	Inactive
OHCPT	4	767.3	0.127	0.11	0.807	
NH2CPT	5	1461.9	0.7756	0.149	0.981	
OHC4CPT	6	1258.6	0.00203	0.0207	0.896	
OHC2CPT	7	1246.4	0.903	0.49905	0.8884	
OHC1CPT	8	1568.3	0.2178	0.1107	0.9066	
OHC3CPT	9	857.27	0.1894	0.0938	0.9113	
OHC5CPT	10	260.77	0.1893	0.051	0.9717	
OMeC1CPT	11	1523.5	5.3209	2.094	0.968	
OMeC2CPT	12	7330.1	377.36	1186	0.976	
OMeC3CPT	13	925.02	0.5962	0.393	0.85	
NNDE-Gly	14	738.41	1.885	1.053	0.906	
MDNNDE-Gly	15	88.169	0.1604	0.2693	5.71E-01	Inactive
Pyrrazalo	16	1259.6	41.271	32.87	0.932	Low Activity
MDCPT(R)	17	924.6	5.3192	3.755	0.8954	
CPT(R)	18					
CMMD-Gly	19	501.28	2.2393	0.7269	0.967	
C2CMMD (RT010)	20	905.18	0.304	0.1766	0.8936	
CMMD (RT006)	21	991.89	0.2016	0.1252	0.8872	

Table 3. Topo I-mediated Cleavage of Supercoiled DNA

B.7.c Reversal of CPT Analog Induced DNA Cleavage.

We also measured the reversibility of the DNA lesion induced by the CPT analogs. As cellular toxicity is a time-dependent phenomenon, those analogs having a long-lived cleavable complex should be more potent antitumor agents. Figure 3 shows the reveral for CPT (S), MDCPT(S), and CMMD. In general, the reversal of CPT follows an exponential decay process, and after 1 hr, <20% of the cleaved DNA remains. In contrast, MDCPT shows an initial quick reversal of ~40% of the cleaved complexes, followed by a much slower linear reversal. Finally, CMMD shows an initial rate of reversal similar to CPT, but only 30-40% of the complexes reverse during this time. These results indicate that those analogs maintaining a larger percentage of cleaved DNA complexes after one hr are somewhat correlated to their cytotoxicty to cancer cell lines.

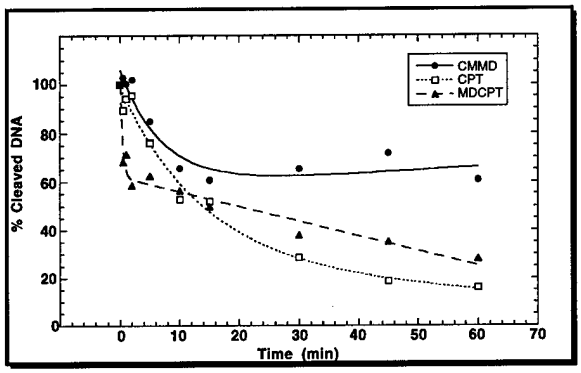


Figure 3. Reversal of Cleaved Complexes for Selected CPT Analogs

The data for complex reversal for all the analogs are given in Table 4. Entries noted † or †† indicate an inactive compound or incomplete data as of this report, respectively. The parameters are according to equation 3 above.

Cpd. Abrev.	C	error	A	error	k	error	B	error	R value
CPT(S)	24.1	33.35	74.6	32.47	0.07	0.037	-0.164	0.559	0.992
MDCPT(S)	62.18	2.68	37.36	5.3	2.643	1.01	-0.616	0.086	0.983
CPT-11	†								
OHCPT	18.8	21.49	87.36	20.89	0.073	0.021	0.0102	0.365	0.996
NH2CPT	83.82	3.57	16.37	5.41	0.763	0.59	-0.04	0.103	0.956
OHC4CPT	42.74		57.32		3.77E-07	305.9	-0.808	17533	0.957
OHC2CPT	76.5	1.44	22.66	2.344	1	0.237	-0.565	0.042	0.995
OHC1CPT	24.78	8.46	66.39	8.381	0.225	0.072	-0.024	0.197	0.985
OHC3CPT	66.68	4.171	29.83	4.102	0.216	0.074	-0.661	0.096	0.996
OHC5CPT	††								
OMeC1CPT	84.51	2.803	17.66	3.703	0.509	0.272	-0.841	0.077	0.993
OMeC2CPT	44.44	23.43	57.88	22.68	0.082	0.043	-0.241	0.418	0.99
OMeC3CPT	49.74	3.71	49.92	7.33	2.593	1.01	-0.487	0.119	0.972
NNDE-Gly	62.15		55.3		3.79E-05	238.9	0.616	13201	0.753

MDNNDE-Gly	†								
Pyrrazalo	†								
MDCPT(R)	†								
CPT(R)	†								
CMMD-Gly	48.18		55.89		0.004	4.185	-0.427	210.8	0.912
C2CPT (RT 010)	60.26		54.02			42.71	0.655	2308	0.852
CMMD (RT 006)	57.71	14.47	48.23	13.82	0.142	0.08	0.141	0.304	0.959
Topotecan	17.7	55.48	93.03	54.35	0.059	0.037	0.056	0.855	0.99

Table 4. Parameters for Reversal of CPT-analog Mediated DNA Cleavage.

In general, all the 10,11-methylenedioxy compounds had a greately delayed recovery of topo I activity after diluting with competing DNA. This delay was mediated by two contributions: an initially rapid resealing of some DNA, followed by a slower, linear resealing of the remaining DNA. These data indicate that much of the potency of the 10,11-MD analogs may be due to the slow reversal of cleaved complexes within the cells.

B.8 In vivo activity of CMMD•Gly and MDCPT•Gly.

B.8.a Methods

Female nude mice weighing approximately 20g were implanted s.c. by trocar with fragments of either MDA-231 or MX-1 harvested from s.c. growing tumors in nude mice hosts. When tumors were approximately 5 mm x 5 mm in size (10 days after inoculation for MDA-231 and 12 days after inoculation for MX-1), the animals were pair-matched into treatment and control groups. Each group contained 8 tumored mice, each of which was ear-tagged and followed individually throughout the experiment. The administration of drugs or vehicle began the day the animals are pair-matched (Day 1) and all injections were done i.p.. For MDA-231, MDCPT•Gly was administered at 1 and 0.5 mg/kg on a qdx5 schedule and at 10 and 5 mg/kg on a qdx1 schedule. CMMD•Gly was administered at 7.5 and 3.75 mg/kg on a qdx5 schedule and at 15 and 7.5 mg/kg on a qdx1 schedule. CPT-11 was used as a positive control and was given at 100 mg/kg on a weekly x 3 schedule. For MX-1, MDCPT•Gly was administered at 0.5 and 0.25 mg/kg on a qdx5 schedule and at 5 and 2.5 mg/kg on a qdx1 schedule. CMMD•Gly was administered at 7.5 and 3.75 mg/kg on a qdx5 schedule and at 15 and 7.5 mg/kg on a qdx1 schedule. CPT-11 was used as a positive control and was given at 100 mg/kg on a weekly x 3 schedule.

Mice were weighed twice weekly, and tumor measurements were taken by calipers twice weekly, starting on Day 1. These tumor measurements were converted to mg tumor weight by L^2 x W/2, and from these calculated tumor weights, the termination date was determined. The experiment was terminated when control tumors reached a size of 500 milligrams. Upon termination, all mice were weighed, sacrificed, and their tumors excised. Tumors were weighed, and the mean tumor weight per group was calculated. In this model, the mean treated tumor weight / mean control tumor weight x 100% (T/C) is subtracted from 100% to give the tumor growth inhibition (TGI) for each group.

With these agents, the final weight of a given tumor was subtracted from its own weight at the start of treatment on Day 1. This difference divided by the initial tumor weight is the % shrinkage. A mean % tumor shrinkage was calculated from data from the mice in a group that experienced regressions.

B.8.b In Vivo Results

Results of studies with MDCPT•Gly and CMMD•Gly are shown in Figures 4 and 5. Against, MDA-231, MDCPT•Gly at 1 mg/kg on a qd x 5 schedule was too toxic (8/8 toxic deaths) to evaluate the efficacy of this agent. Using this schedule at 0.5 mg/kg, no antitumor activity was observed. A mean final tumor weight of 2414.5 mg compared to 1959.5 mg in vehicle controls and 507.3 mg in animals treated with CPT-11 resulted. MDCPT•Gly at 10 mg/kg on a qd x 1 schedule was, again, too toxic to evaluate antitumor activity of this agent. When this agent was administered at 5 mg/kg on the qd x 1 schedule, the final tumor weight (673.0 mg; TGI = 68.0) was significantly ($p < 0.05$) decreased compared to controls and was comparable to animals treated with CPT-11 (507.3 mg; TGI = 73.0). There were no partial or complete responses observed with administration of MDCPT•Gly using this tumor model.

CMMD•Gly at 3.75 mg/kg on a qd x 5 schedule was inactive against the MDA-231 tumor model. When this agent was administered at 7.5 mg/kg on the same schedule, modest antitumor activity was observed with a final tumor weight of 1080.5 and TGI of 46.5. CMMD•Gly at 7.5 and 15 mg/kg on a qd x 1 schedule was inactive against this tumor model. No partial or complete responses were noted with CMMD•Gly and the MDA-231 tumor model. This agent was well tolerated with no toxic deaths or substantial change in body weight.

Administration of CPT-11 resulted in a TGI of 73.3 with one partial response of 53%. The final tumor weight following CPT-11 (507.3-CPT-11 versus 1959.5-controls) administration was significantly less than vehicle treated controls.

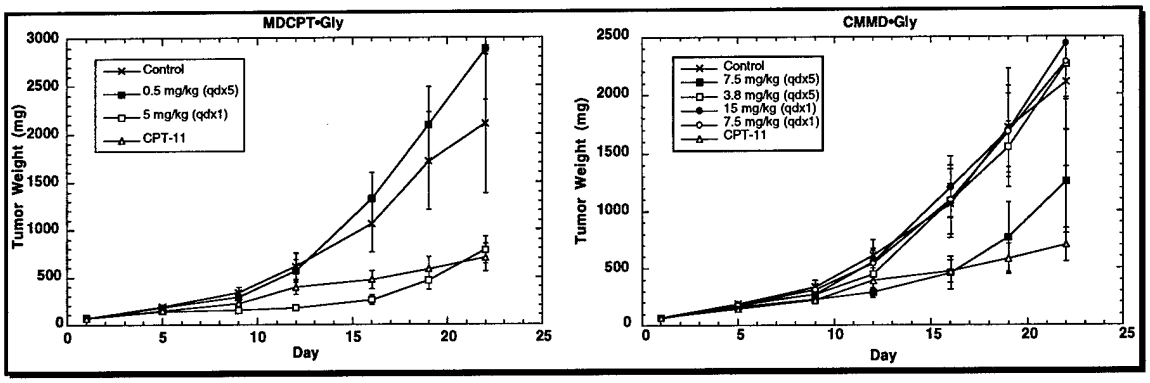


Figure 4. Activity of MDCPT•Gly and CMMD•Gly against MDA-231 Xenografts

MDCPT•Gly and CMMD•Gly both showed considerable antitumor activity against the MX-1 human breast tumor xenograft model (Figure 5). MDCPT•Gly, at 0.25 and 0.5 mg/kg on a qd x 5 schedule, resulted in 3/8 and 8/8 complete responses, respectively. MDCPT•Gly at 5 mg/kg on a qd x 1 schedule produced 7 complete responses while one complete response was noted at 2.5 mg/kg using the same dosing schema. MDCPT•Gly was, in general, well tolerated with one toxic death at 5 mg/kg on a qd x 1 schedule.

CMMD•Gly, at 3.75 and 7.5 mg/kg on a qd x 5 schedule, resulted in complete responses in all animals. When this agent was administered on the qd x 1 schedule, 3 and 1 complete responses were seen at 7.5 and 15 mg/kg, respectively. The mean final tumor weight was 868.4 mg at 7.5 mg/kg and was 177.5 mg at 15 mg/kg (controls-1227.4 mg) using the qd x 1 schedule. CMMD•Gly was well tolerated with no toxic deaths or substantial change in body weight.

CPT-11 (100 mg/kg) was used as a positive control and produced 7/8 complete responses using a weekly x 3 schedule.

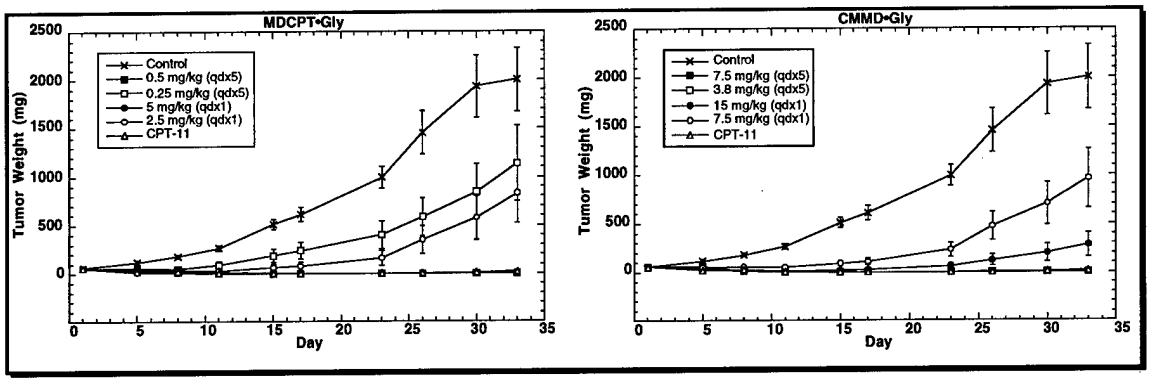


Figure 5. Activity of MDCPT•Gly and CMMD•Gly against MX-1 Xenografts

B.8.c Discussion of in vivo Results

Two glycinate esters of the topoisomerase I-active analogs CMMD and MDCPT were evaluated in vivo using the MX-1 and MDA-231 human breast tumor xenograft models. MDCPT•Gly and CMMD•Gly were administered on either a qd x 5 or qd x 1 schedule at various doses. Final tumor weight, tumor growth inhibition, and partial or complete responses were the endpoints of this study that evaluated the efficacy of these new agents.

Using the MX-1 breast tumor model, both agents, regardless of schedule, appeared active, producing a substantial number of complete responses. There was a trend toward an increase in observed responses seen when these agents were administered on a qd x 5 schedule. Since all agents, including CPT-11, had substantial activity against this tumor model it is difficult to determine which was more efficacious. The antitumor activity of these agents must take into consideration the frequently noted high susceptibility of the MX-1 tumor model to a wide-range of chemotherapeutic agents.

These agents had considerably less activity against the MDA-231 human breast tumor xenograft model. Toxicity of MDCPT•Gly on both schedules of administration limited our ability to detect antitumor activity of this compound. MDCPT•Gly at 5 mg/kg (qd x 1) did appear to be active by National Cancer Institute standards with a TGI > 58%. CMMD•Gly had modest activity at 7.5 mg/kg (qd x 5), however, its TGI was less than 58%. Using a qd x 1 schedule, CMMD•Gly was inactive against this model. Both MDCPT•Gly and CMMD•Gly were less active than CPT-11 using this tumor model.

C. CONCLUSIONS

We evaluated a series of CPT analogs using several biochemical tests. Our results indicate that the MDCPT analog was as effective as the CMMD analog in inducing topo I-mediate cleavage of DNA. Both analogs showed slow reversal of the topo I-mediated complexes, but with CMMD reversing to a somewhat lesser degree than MDCPT. Against three breast cancer cell lines, both compounds were highly active. We also noted that other 7- or 10-substituted analogs of MDCPT did not significantly enhance the activity in any of these tests.

We then used the glycinate esters of MDCPT and CMMD in our *in vivo* assays. Both compounds were highly effective against the MX-1 xenograft model, and both were reasonably effective against the MDA-231 xenograft. Due to the aqueous instability of CMMD (the 7-chloro group is hydrolyzed with time), we feel that the MDCPT is the best choice among the analogs examined to take forward for clinical testing. We are currently pursuing moving the MDCPT•Gly to clinical studies, as well as pursuing the goals of year 3 of this award.

D. Progress toward Statement of Work

According to our original statement of work (Appendix 7 in our grant proposal; copy attached), the following progress has been made. Note that year 4 of the grant, primarily concerning nititdine analogs, was deleted by the reviewers.

Goal	Research Triangle Institute	Institute for Drug Development
1	Completed	Completed (actually developed by RTI)
2	Completed	Completed
3	Completed	Completed (only Glycinate compounds were examined)
4	Partially completed (Pyrrazalo compound synthesized)	In progress
5	Partially completed (B-1 and B-2 synthesized. B-4 in preparation)	Partially completed (Pyrrazalo compound inactive)
6	No active compounds identified	In progress
7	Goal deleted by reviewers	No active compounds identified
8	In progress	Goal deleted by reviewers
9	In progress	In progress

E. REFERENCES

1. Wall, M. E., Wani, M. C., Cook, C. E., Palmar, K. H., MacPhail, A. T., and Sim, G. A. (1966) Plant antitumor agents. 1. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminata.* **88,** 3888-3890.
2. Gottlieb, J. A., Guarino, A. M., Call, J. B., Oliverio, V. T., and Block, J. B. (1970) Preliminary pharmacologic and clinical evaluation of camptothecin sodium (NSC 100880) **54,** 461-470.
3. Muggia, F. M., Creaven, P. J., Hansen, H., Cohen, M. H., and Selawry, O. S. (1972) Phase I clinical trial of weekly and daily treatment with camptothecin (NSC 100880): Correlation with preclinical studies **56,** 515-521.
4. Creaven, P. J., Allen, L. M., and Muggia, F. M. (1972) Plasma camptothecin (NSC-100880) levels during a 5-day course of treatment: Relation to dose and toxicity **56,** 573-578.
5. Hsiang, Y. H., Hertzberg, R., Hecht, S., and Liu, L. F. (1985) Camptothecin induced protein-linked DNA breaks via mammalian DNA topoisomerase I **260,** 14873-14878.
6. Hsiang, Y.-H., Liu, L. F., Wall, M. E., Wani, M. C., Kirschenbaum, S., Silber, R., and Potmesil, M. (1989) DNA topoisomerase I-mediated DNA cleavage and cytotoxicity of camptothecin analogs **49,** 4385-4389.
7. Champoux, J. (1990) Mechanistic aspects of type-I topoisomerases, Cold Spring Harbor Laboratory, Cold Spring Harbor.
8. Wang, J. C. (1991) DNA topoisomerases: why so many **266,** 6659-6662.
9. Holm, C., Covey, J. M., Kerrigan, D., and Pommier, Y. (1989) Differential requirement of DNA replication for the cytotoxicity of DNA topoisomerase I and II inhibitors in Chinese hamster DC3F cells **49,** 6365-6368.
10. Hsiang, Y.-H., Lihou, M. G., and Liu, L. F. (1989) Arrest of DNA replication by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin **49,** 5077-5082.
11. Pommier, Y., Leteurtre, F., Fesen, M., Fujimori, A., Betrand, R., Solary, E., Kohlhagen, G., and Kohn, K. W. (1994) Cellular determinants of sensitivity and resistance to DNA topoisomerase inhibitors **12,** 530-542.
12. Kawato, Y., Aonuma, M., Hirota, Y., Kuga, H., and Sato, K. (1991) Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11 **51,** 4187-4191.
13. Kunimoto, T., Nitta, K., Tanaka, T., Uehara, N., Baba, H., Takeuchi, M., Yokokura, T., Sowada, S., Miyasaka, T., and Mutai, M. (1987) Antitumor activity of 7-ethyl-10-[4-piperidino)-1-piperidino]carbonyloxy-camptothecin, a novel water-soluble derivative of camptothecin against murine tumors **47,** 5944-5947.
14. Slichenmyer, W. J., Rowinsky, E. J., Donehower, R. C., and Kaufman, S. H. (1993) The current status of camptothecin analogues as antitumor agents **85,** 271-291.
15. Von Hoff, D. D., Casper, J., Bradley, E., Sandbach, J., Jones, D., and Makuch, R. (1981) Association between human tumor colony forming assay results and response of an individual patient's tumor to chemotherapy **70,** 1027-1041.
16. Burris, H. A., III, Awada, A., Kuhn, J. G., Eckardt, J. R., Cobb, P. W., Rinaldi, D. A., Fields, S., Smith, L., and Von Hoff, D. D. (1994) Phase I and pharmacokinetic studies on topotecan administered as a 72 or 120 h continuous infusion **5,** 394-402.

APPENDIX 1.

CHART C2

Synthesis of E Ring Ketone Analog of CPT

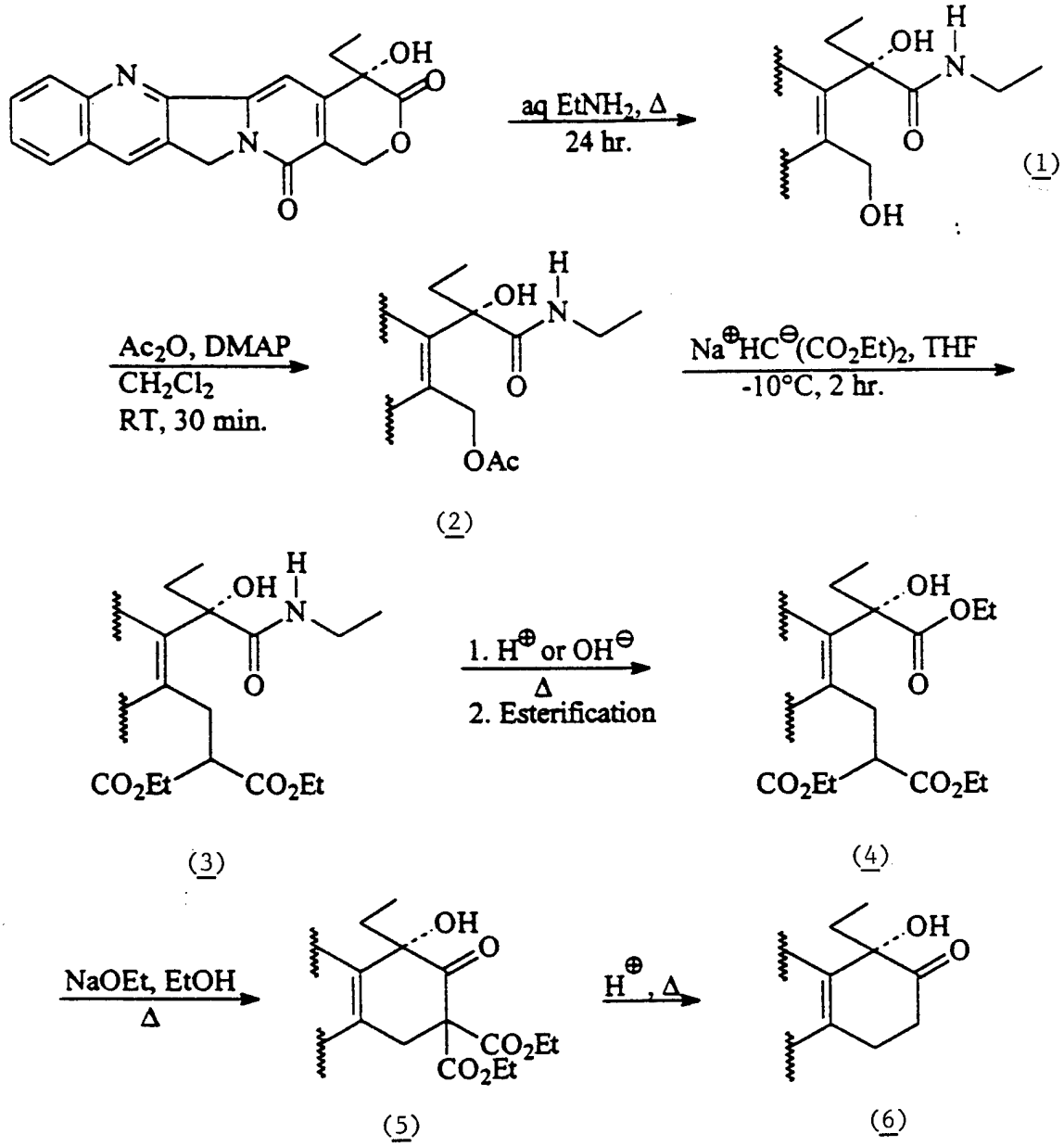
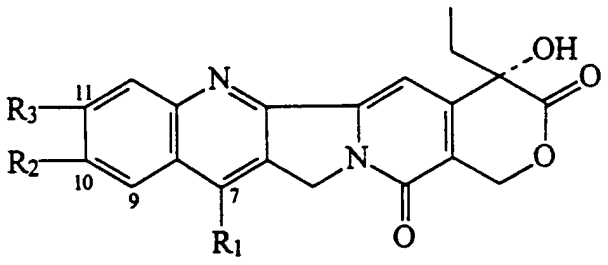


Chart 1



Camptothecin (CPT) $\xrightarrow{1\ step}$ 7-Hydroxymethyl-CPT, R_2=R_3=H, R_1=CH_2OH

$\downarrow$ 1 step

(1) 7-Bromomethyl-CPT, R_2=R_3=H, R_1CH_2Br

10-Hydroxy-CPT,
R_1=R_3=H, R_2= -OH $\longrightarrow$ 10-Hydroxy-7-hydroxymethyl
R_3=H, R_2=OH, R_1=CH_2OH

(2) 10-Hydroxy-7-bromomethyl-CPT
R_3=H, R_2=OH, R_1=CH_2Br

10,11-Methylenedioxy-CPT,
R_3=R_2=  , R_1= H $\longrightarrow$ 7-hydroxymethyl-10,11-MD-CPT
R_3=R_2=  , R_1=CH_2OH

$\swarrow$

7-Chloromethyl-10,11-MD-CPT
R_3=R_2=  , R_1=CH_2Cl $\xleftarrow{1\ step}$ (3) 7-Bromomethyl10,11-MD-CPT

Chart 2

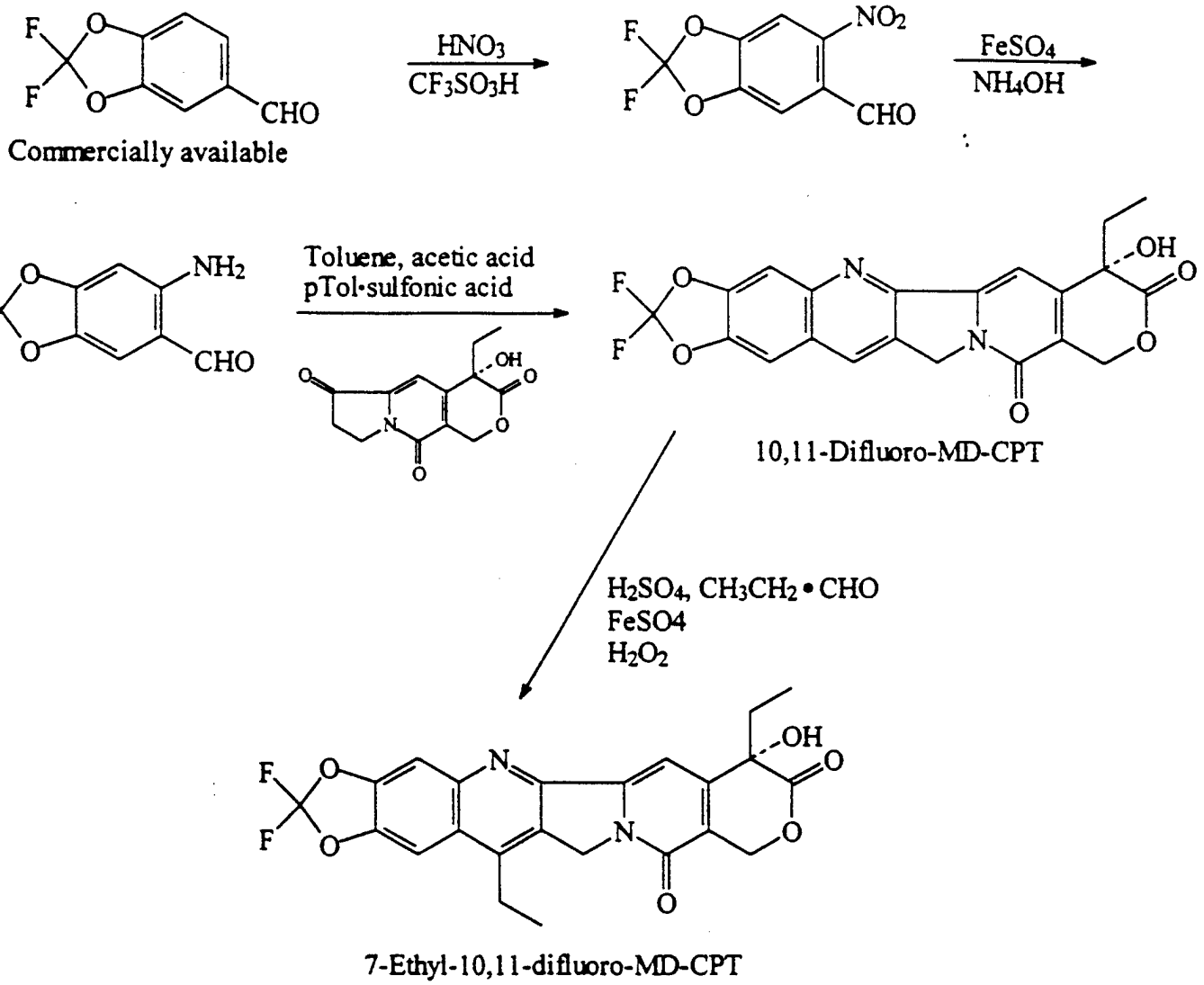
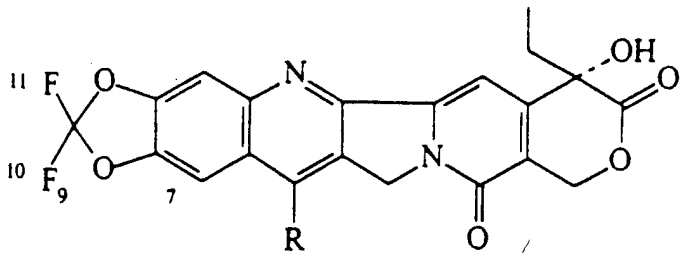

HNO3
CF3SO3H
FeSO4
NH4OH
Commercially available
Toluene, acetic acid
pTol·sulfonic acid
10,11-Difluoro-MD-CPT
H2SO4, CH3CH2•CHO
FeSO4
H2O2
7-Ethyl-10,11-difluoro-MD-CPT

Chart 3



1. Difluoro-10,11-methylenedioxy (DFMD)-20(S)-camptothecin (CPT) R=H
2. 7-Ethyl-DFMD-CPT, R=C_2H_5
3. 7-Chloromethyl-DMD-CPT, R=CH_2Cl
4. 7-Bromomethyl-DMD-CPT, R=CH_2Br
5. **1** $\xrightarrow{HNO_3}$ 9-Nitro-DFMD-CPT, R=H
6. **5** $\xrightarrow{SnCl_2}$ 9-Amino-DFMD-CPT, R=H

APPENDIX 2

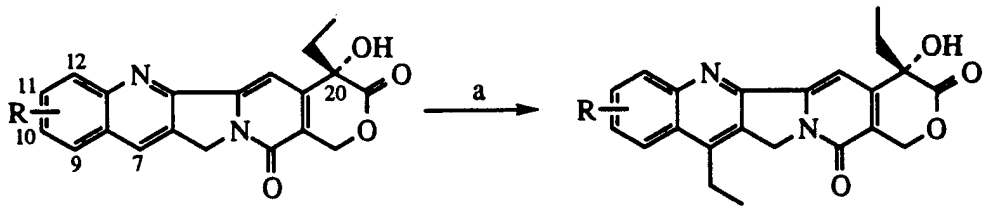
Statement of Work

The proposal will be well coordinated between RTI (Research Triangle Institute) and IDD (Institute for Drug Development). We will prepare and evaluate the biological activity of the compounds according to the table. The data will be sent immediately to Dr. Wall at the RTI. Conference calls will be made frequently between Drs. Wall and Chen to facilitate and identify any new findings. Compounds to be scaled up will be determined by the PI and all of the investigators. A statistical analysis will be performed annually or as needed.

Months	Reseach Triangle Institute		Institute for Drug Development	
1-4	1	Prepare additional quantities[a] of compounds **A-1** through **A-6** (Chart A)	1	Establish HPLC procedure for the separation of selected 20(S)-O-Gly and 20(S)-OH CPT
5-8	2	Prepare additional quantities[a] of compounds **A-7** through **A-12** (Chart A)	2	Determine whether 20(S)-O-Gly CPT analogs are active compounds or pro-drugs
9-12	3	Prepare 250 mg of the 20(S)-O-Gly of **B-1** (7-chloromethyl-10,11-MD 20(S)-OH) in Chart B; Prepare small quantities of **D3a** and **D-3b** (Chart D1)	3	Evaluate selected pairs of 20(S)-OH and 20(S)-O-Gly against human breast xenografts
13-16	4	Prepare small quantities[b] of **A-11** through **A-17** (Chart A); Initiate synthesis of E-ring ketone analog **C-10** (Chart C)	4	Perform a statistical analysis to correlate lactone stability with biological data based on preliminary data
17-20	5	Prepare small quantities of **B-2** through **B-4** (Chart B); Prepare additional quantities of compounds **D-14** and **D-16.**	5	Determine whether compounds **A-11** through **A-17** have potent *in vitro* activity; Evaluate selected agents *in vivo*
21-24	6	Prepare additional quantities of 20(S)-glycinates of the best compounds of **A-11** through **A-17**	6	Evaluate the biological activity of compounds **B-2** through **B-4.**
25-28	7	Prepare small quantities of **D-7a** through **D-7d**	7	Evaluate the glycinates of **A-11** through **A-17** (only on the active **A-11** through **A-17**)
29-32	8	Prepare small quantities of **B-5** through **B-10**	8	Evaluate the nitidine analogs **D3a, D-3b, D-14, D-16;** Evaluate selected agents *in vivo*
13-36	9	Complete multi-step syntheses of E-ring Ketone analog **C-10** (Chart C)	9	Discuss with RTI the selection of additional compounds for scale up. Evaluate compounds **B-5** through **B-10**
37-44	10	Prepare small quantities of **D-7e** through **D-7i;** Scale up potential compounds for *in vivo* evaluation	10	Evaluate Compound **C-10;** Perform a statistical analysis; Evaluate selected agents *in vivo*
41-44	11	Prepare multi-step syntheses of analogs **D-11a** through **D-11d**	11	Evaluate nitidine analogs **D-7a** through **D-7i.**
44-48	12	Prepare additional CPT and nitidine analogs as needed	12	Evaluate nitidine analogs **D-11a** through **D-11d**

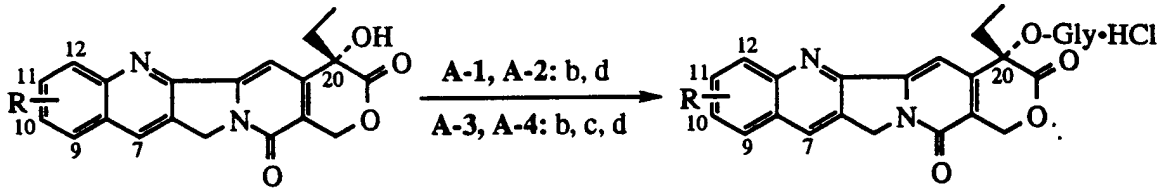
[a]Additional quantities (50-250 mg) depending on the doses of the compounds to be used in animal studies;
[b]Samll quantities (5-10 mg).

III.B.2. Synthesis of CPT Analogs with Non-Covalent Substituents (Chart A)



A-1, R=10,11-OCH_2O- **A-2**, R=10,11-OCH_2O-

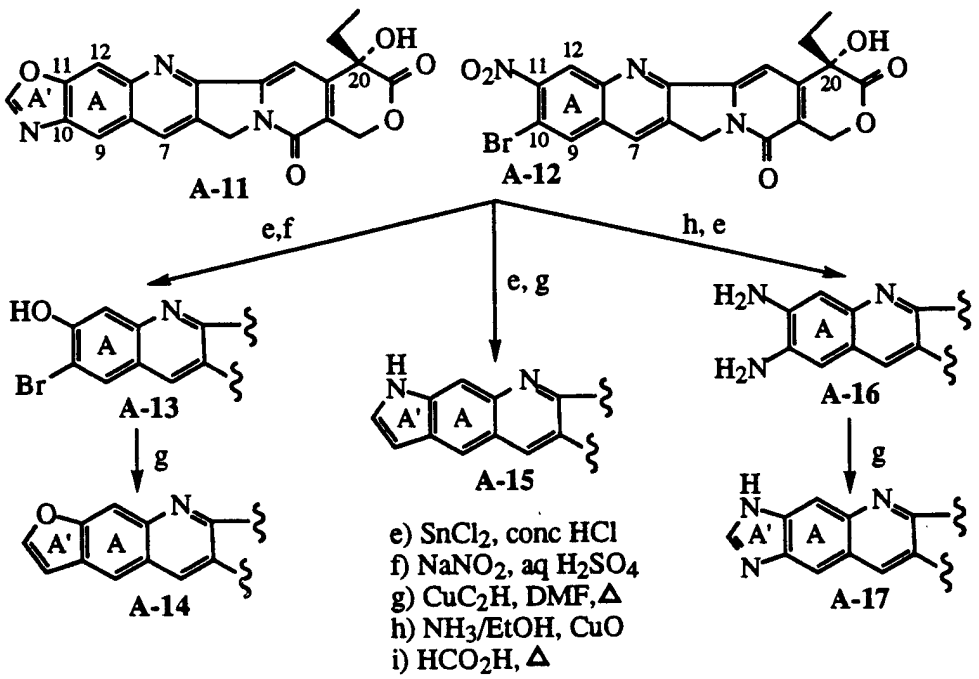
a) CH_3CH_2CHO, aq H_2SO_4, $FeSO_4$, aq H_2O_2
0°C ⟶ RT, 2 hr



A-1, R=10,11-OCH_2O-
A-2, R=7-Et-10,11-OCH_2O-
A-3, R=9-NO_2-10,11-OCH_2O-
A-4, R=7-Et-9-NO_2-10,11-OCH_2O-
A-5, R=9-NH_2-10,11-OCH_2O-
A-6, R=7-Et-9-NH_2-10,11-OCH_2O-

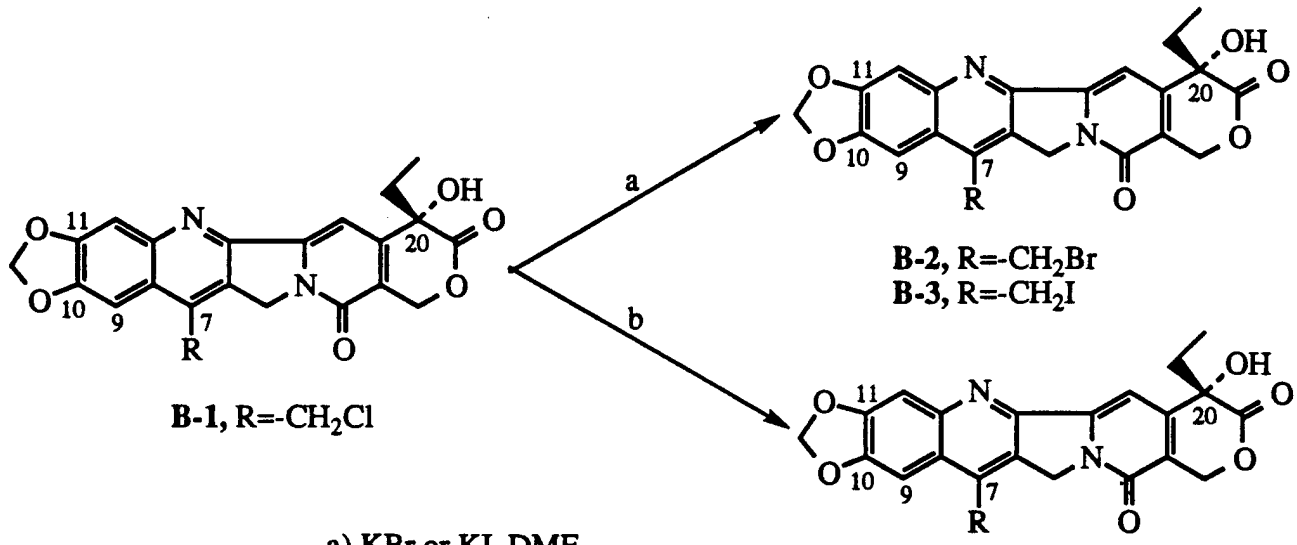
A-7, R=10,11-OCH_2O-
A-8, R=7-Et-10,11-OCH_2O-
A-9, R=9-NH_2-10,11-OCH_2O-
A-10, R=7-Et-9-NH_2-10,11-OCH_2O-

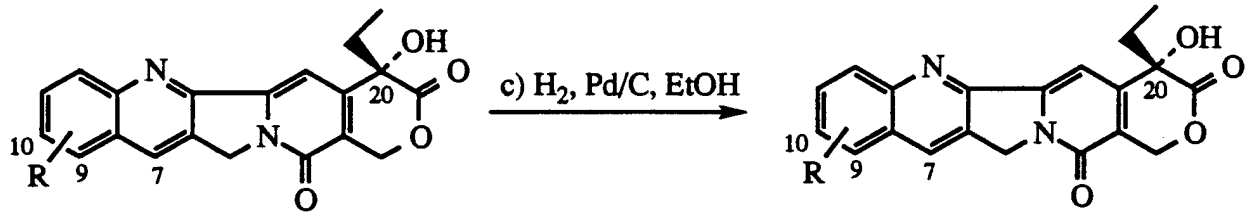
b) t-BOC glycine, DMAP, DCC, CH_2Cl_2/DMF, < 0°C⟶ RT
c) H_2, Pd/C, EtOH, RT, 1 atm (Conditions apply for A-3 and A4 only)
d) HCl/dioxane, CH_2Cl_2, 0°C ⟶ RT

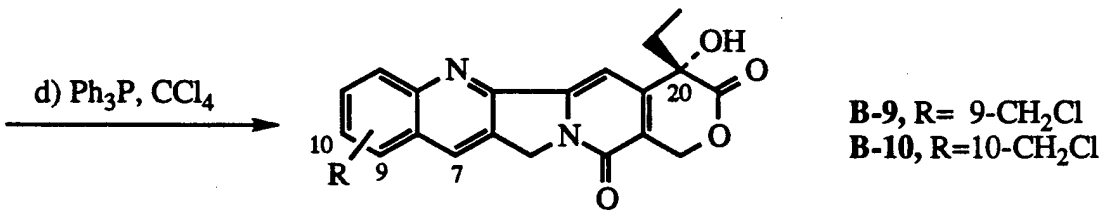


e) $SnCl_2$, conc HCl
f) $NaNO_2$, aq H_2SO_4
g) CuC_2H, DMF, Δ
h) NH_3/EtOH, CuO
i) HCO_2H, Δ

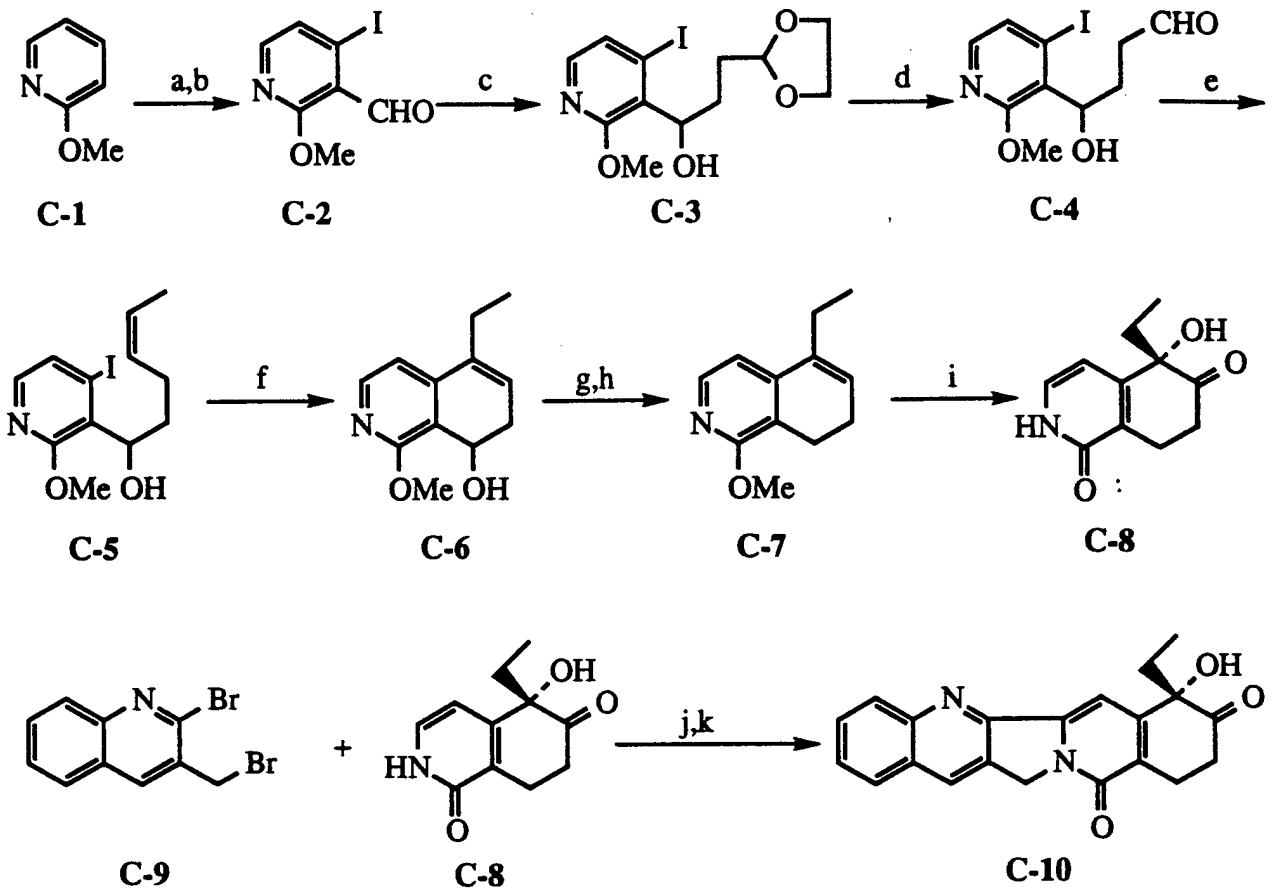
III.B.3. Synthesis of CPT analogs with Covalent Substituents (Chart B)

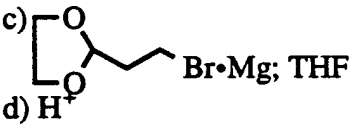






III.B.4. Synthesis of CPT analog with E-Ring Ketone (Chart C)



a) t-BuLi, RNCHO (R=alkyl)
b) n-BuLi; I_2
c)  Br•Mg; THF
d) H^+
e) $CH_3CH=P(C_6H_5)_3$
f) $Pd(OAc)_2$
g) Thiocarbonyldiimidazole, DMAP
h) Bu_3SnH, AIBN
i) Sharpless oxidation; I_2, $CaCO_3$
J) t-BuOK
k) $Pd(OAc)_2$